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16. ABSTRACT

Acute exposure to trimethyltin (TMT) produces alterations in hippocampal morphology. The purpose of this study was to arrive at a simple method for quantitative assessment of the gross changes in morphology which could then be used as a correlate in studies of TMT toxicity. Adult Long-Evans male hooded rats were treated with a single dose of TMT chloride and sacrificed either (a) within 11 days; (b) following 30 days; or (c) 105 days following treatment. Among a variety of morphological measures explored, the easiest and most clearly dosage-related was length of the line of pyramidal cells, from CA1 through CA3c. TMT shortened this line in a dosage and time since treatment increased. It was concluded that this measurement may provide a useful morphological correlate for physiological and behavioral studies of TMT toxicity.

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Trimethyltin-Induced Changes in Gross Morphology of the Hippocampus[1]

ROBERT S. DYER, TERESA L. DESHIELDS[2] AND WILLIAM F. WONDERLIN[3]

Neurophysiology Branch, Neurotoxicology Division, U. S. Environmental Protection Agency
Research Triangle Park, NC 27711

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Hippocampus Hippocampal degeneration Trimethytin Organotin Trimethyltin chloride

ACUTE exposure to trimethyltin (TMT) produces alterations in hippocampal morphology [2, 4, 7], but there has been no quantitative assessment of hippocampal damage as a function of TMT dosage. Since a large proportion of current studies of TMT toxicity have explored behavioral and physiological parameters presumed sensitive to hippocampal dysfunction, a quantitative description of gross changes in hippocampal morphology might be useful to correlate for relating neuropathological changes to alterations in these endpoints. Thus, the main intent of the present study was to arrive at a simple gross measure of TMT-induced hippocampal damage which could be used as a correlate in other studies. Availability of such a measure will allow future studies to determine the relationship between the primary endpoints measured and a simple measure of hippocampal damage. It is not presumed that perfect correlations will be obtained between crude morphological measures and behavioral or physiological parameters. On the other hand it is unreasonable to expect behavioral and physiological studies to routinely provide detailed morphological assessment of hippocampal damage. Thus, a simply made measure has merit as a compromise between no assessment and detailed assessment for relating two such data sets.

Brains used for the present study were obtained from three studies completed for other purposes. In two of these studies, electrodes were implanted unilaterally in the hippocampus for purposes of either stimulation or recording. In the other study, only behavioral measures were obtained. Assessment of brains from these two different types of experiments allowed determination of the role played in hippocampal degeneration by repeated electrical stimulation and/or electrode placement.

METHOD

Adult male Long-Evans hooded rats, obtained from Charles River Breeding Company, were housed individually in plastic cages with wood chip bedding and unless otherwise indicated given free access to food and water throughout their stay in the lab. Trimethyltin chloride (TMT) (ICN, Plainview, NY) was administered by gavage in a volume of 1 ml/kg, and dosages were either 0 (saline control), 5, 6, or 7 mg/kg given as the base.

In the first experiment, 50 rats surgically implanted with electrodes in the left dorsal hippocampus (see hippocampal afterdischarge study in Dyer *et al.*, pp. 203–208, this volume) were used. The animals were divided into groups accordingly: unstimulated untreated controls (n=10), saline treated controls (n=10), 5 mg/kg TMT (n=10); 6 mg/kg TMT (n=10) and 7 mg/kg TMT (n=10). Thirty days following treatment, the rats were perfused through the heart with saline followed by 10 percent neutral buffered Formalin saturated with potassium ferrocyanide (used for prussian blue electrode placement test). Frozen sections (80 μM) were ob-

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[2]Present address: Department of Psychology, University of Georgia, Athens, GA.
[3]Present address: Department of Environmental Health Sciences, The Johns Hopkins University, 615 North Wolfe Street, Baltimore, MD.

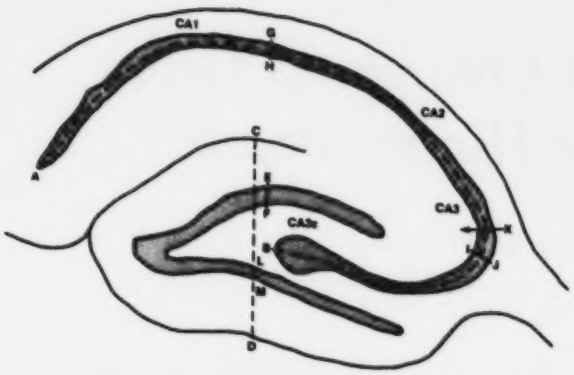

FIG. 1. Diagrammatic sagittal section through the rat hippocampal formation illustrating measurements made. (A–B) length of the pyramidal cell line; (C–D) thickness of the dentate gyrus; (E–F) thickness of the granule cell layer; (G–H) thickness of CA1; (I–J) thickness of CA3; (K) likely area for "density" measurement to have been made; (C–E) and (M–D) thickness of molecular layer; (F–L) intrahilar thickness.

tained in the sagittal plane. At least 5 sections on the left side of the brain were obtained through the segment containing the electrode tract (about 2.5 mm lateral). At least 5 sections were obtained from the right side of the brain about 2.0 mm lateral to the midline. Obtaining these sections from a more medial location than the left side allowed determination of whether lateral or medial areas of the dorsal hippocampus were more sensitive to TMT. Sections were stained with cresyl violet.

The measurements made are illustrated in Fig. 1. These measurements, which were chosen to quantify changes suggested by casual observation of a few brains from animals receiving high dosages, included: (1) a measurement of the length of the pyramidal cell line, from the CA1 subiculum border until the organized line of pyramidal cells was no longer easily discriminated (end of CA3c in normal tissue) (A to B in Fig. 1); (2) thickness of the dentate gyrus, measured by drawing a perpendicular from the hippocampal fissure to the ventral most tissue of the fascia dentata (C to D in Fig. 1); (3) thickness of the dentate granule cell layer, measured by drawing a perpendicular through the granule cell layer at the thickest extent of either the dorsal or ventral blade (E to F in Fig. 1); (4) thickness of the CA1 pyramidal cell layer, measured by drawing a perpendicular through the layer at the thickest extent of its horizontal course (G to H in Fig. 1); (5) thickness of the CA3 pyramidal cell layer, measured by drawing a perpendicular through this cell layer at the point closest to the fornix (I to J in Fig. 1); and (6) density of cells in CA3, measured by drawing a perpendicular through CA3 at its least dense area (subjectively determined (e.g., at K in Fig. 1) and counting the number of pyramidal cells touched by the line as it pierced the layer. All of these measurements were made in a blind fashion, and each measurement was made on five sections from each side of the brain. Section order was maintained to allow assessment of lateral distance from the midline within left and right blocks of sections.

Measures (1) and (2) above were made by projecting the sections onto plain white paper (1 mm=37 μm) and measuring to the nearest 1 mm. Measure (6) was made at 450×, and all other measures were made to the nearest 5 μ at 100×.

In the second experiment, rats were implanted with electrodes in the left dorsal hippocampus and, one week later, treated with either saline (n=4) or 6 mg/kg TMT (n=9). Saline-treated animals were sacrificed 6–8 days following treatment, and TMT-treated animals were sacrificed either 3–5 (n=2), 6–8 (n=5) or 9–11 (n=2) days following treatment. Sections were obtained as described for the first experiment, but measurements were only made upon sections from the right side of the brain. At least 5 sections were measured for each animal, beginning 2.5 mm lateral to the midline, and these values were then averaged within animal. Since the first (30-day post-treatment sacrifice time) study (see Results) indicated that length of the pyramidal cell line was most affected by TMT, only this measurement was made in the second (<11-day post-treatment sacrifice time) study.

In the third experiment, rats were not implanted with electrodes. They were food deprived, trained on a radial arm maze task, and treated with either 0 (n=7) or 6 (n=8) mg/kg TMT. Animals were then retrained on the task (18) and perfused 105 days following treatment. Frozen sagittal sections were obtained from one hemisphere (40 μm) beginning about 3.0 mm lateral to the midline. At least 6 sections were taken from each brain, but, based upon the absence of a treatment × section interaction in the first experiment (see Results), only 1 section was measured for each animal. In addition to the measurements made in the first study, measurements were made of the length of the line of dentate granule cells (dorsal and ventral blades), the thickness of the molecular layer of the fascia dentata (dorsal and ventral blades (C to E and M to D in Fig. 1)), and of the hilar region of the fascia dentata (F to L in Fig. 1) in order to determine the origin of changes observed in the measurement obtained for overall thickness of the dentate gyrus. In this study, the measurement point within the dentate gyrus was the midpoint of the granule cell layer from the opening of the hilus to the elbow. Measurements of CA1 layer thickness and CA3 thickness and density were not made in this study.

Data from each experiment were analyzed using multivariate analysis of variance (MANOVA) techniques. Significant MANOVAs were followed by repeated measures ANOVAs with section number as the repeated measure. Nonhomogeneity of variance-covariance matrices was assumed, and the Geisser-Greenhouse correction was therefore applied [8]. In addition, a Bonferroni correction [6] was applied to correct for the number of ANOVAs performed in each experiment. For the first experiment, 3 sets of MANOVAs were performed (α=0.05/3=0.017). One MANOVA was performed for the left side measurements and one for the right side measurements. Since there were 6 measurements made on each side of the brain, there were 6 ANOVAs to be performed if a MANOVA was significant. The required level of significance for each ANOVA was therefore 0.05/6 =0.0083. Testing for effects of section number allowed assessment of the influence of small variations in distance from the midline upon the measurements, as well as any section number × dosage interactions. To make similar assessments for a wider range of lateral distance, the values obtained for the five sections were averaged for each side for each animal. The third MANOVA was performed upon this left (lateral) versus right (medial) data set. The same strategy was adopted for analysis of the other experiments. Significant ANOVAs were followed by Duncan's multiple range test for individual comparisons (α=0.05). Using these procedures the experiment-wise error rate was maintained at 0.05.

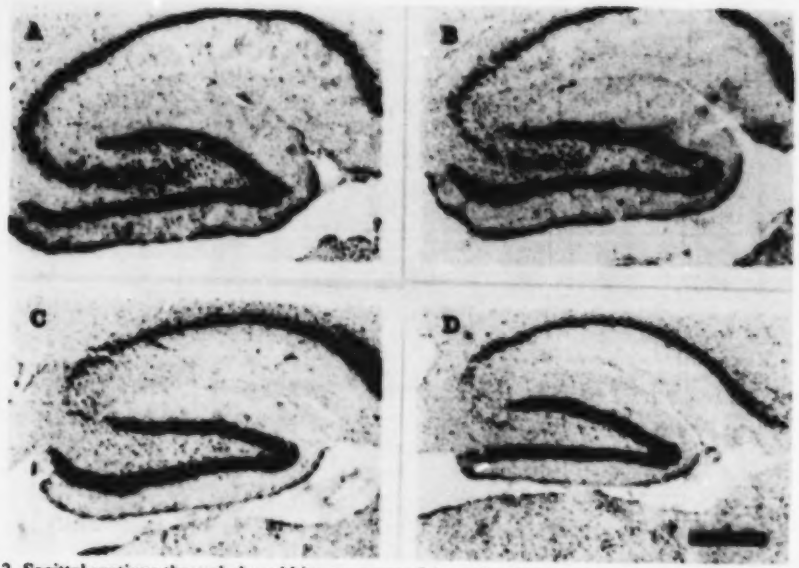

FIG. 2. Sagittal sections through dorsal hippocampus of 4 rats for Experiment 1. Sacrifice time was 30 days following treatment. (A) control; (B) 5 mg-kg TMT; (C) 6 mg/kg TMT; (D) 7 mg/kg TMT. Calibration bar: 0.5 mm.

RESULTS

In general, the results confirmed previous observations that the pyramidal cells were most sensitive to TMT, as measured by cell loss. Figure 2 illustrates typical sagittal sections obtained from animals in the first experiment. Measurements obtained from the first experiment indicated that length of the pyramidal cell line was sensitive to TMT treatment. Thickness of the dentate gyrus was also decreased by TMT, as was density of cells within CA3, (all F's $(4,43)>4.38$, all p's<0.005). On the other hand, neither thickness of the granule cell layer, thickness of CA3 or thickness of CA1 were significantly affected by the TMT treatment, all F's $(4,43)<3.06$, all p's>0.03. The TMT treatment × section interaction tested whether, within blocks of medial (right side) or lateral (left side) sections, there was an influence of distance from the midline on presence, magnitude or direction of treatment effect. The analysis indicated that there were no significant treatment × section interactions, F's $(4,43)<1.77$, p's>0.15.

Figure 3A illustrates that while there were no differences between the implanted but untreated, unstimulated controls and the rats receiving saline, there was a dose-dependent decrease in length of the pyramidal cell line following TMT administration. The Duncan's multiple range tests indicated that for the right side the unstimulated and control groups did not differ from each other. The 6 and 7 mg/kg TMT groups did not differ from each other, but did differ from all other groups, and the 5 mg/kg group differed significantly from the unstimulated group but not from the 0 mg/kg control group. The same relationships held for the left side, except that the 5 mg/kg TMT group differed from both the saline control and the unstimulated control. In addition, the 6 and 7 mg/kg groups differed from each other. Figure 3B illustrates the effect of TMT upon thickness of the dentate gyrus. As with length of the pyramidal cell line, TMT reduced the measured values. In this case, however, the significant paired comparison differences were only found on the left side. Here the 7 mg/kg group differed significantly from all groups except the 6 mg/kg group. The 6 mg/kg group differed from both control groups, but not from the 5 mg/kg group. The 5 mg/kg group did not differ from either control group.

Figure 3C illustrates the effect of TMT upon density of cells within CA3. TMT reduced the density of cells within CA3, but only in the 6 and 7 mg/kg groups, and only on the right side measurements. The significant overall ANOVA was not accompanied by any significant individual paired comparisons on the left side. Figure 3D-F indicates the non-significant effects of TMT upon the other measurements obtained in this experiment.

Although there were no significant treatment by section interactions, there were significant effects of section, thus indicating that within blocks of left and right sections some of the measurements taken depended upon distance from the midline. Length of the pyramidal cell line increased with distance from the midline in both medial (right) and lateral (left) blocks of sections. In the medial block of sections, the thickness of the dentate gyrus increased and thickness of the CA1 cell layer decreased with distance from the midline, all F's $(1,43)>10.46$, all p's<0.002. No other measurements were significantly affected by section number, all F's $(1,43)<2.15$, all p's>0.15.

From Figure 3, it is evident that in most cases, differences existed between the medial and lateral blocks of sections even when no differences existed between sections within block. The third MANOVA supported the medial versus lateral block differences, $F(6,81)=29.64$, $p<0.0001$. Reference to Fig. 3 indicates the nature of the differences. Those which were significant were: density of CA3, thickness of the

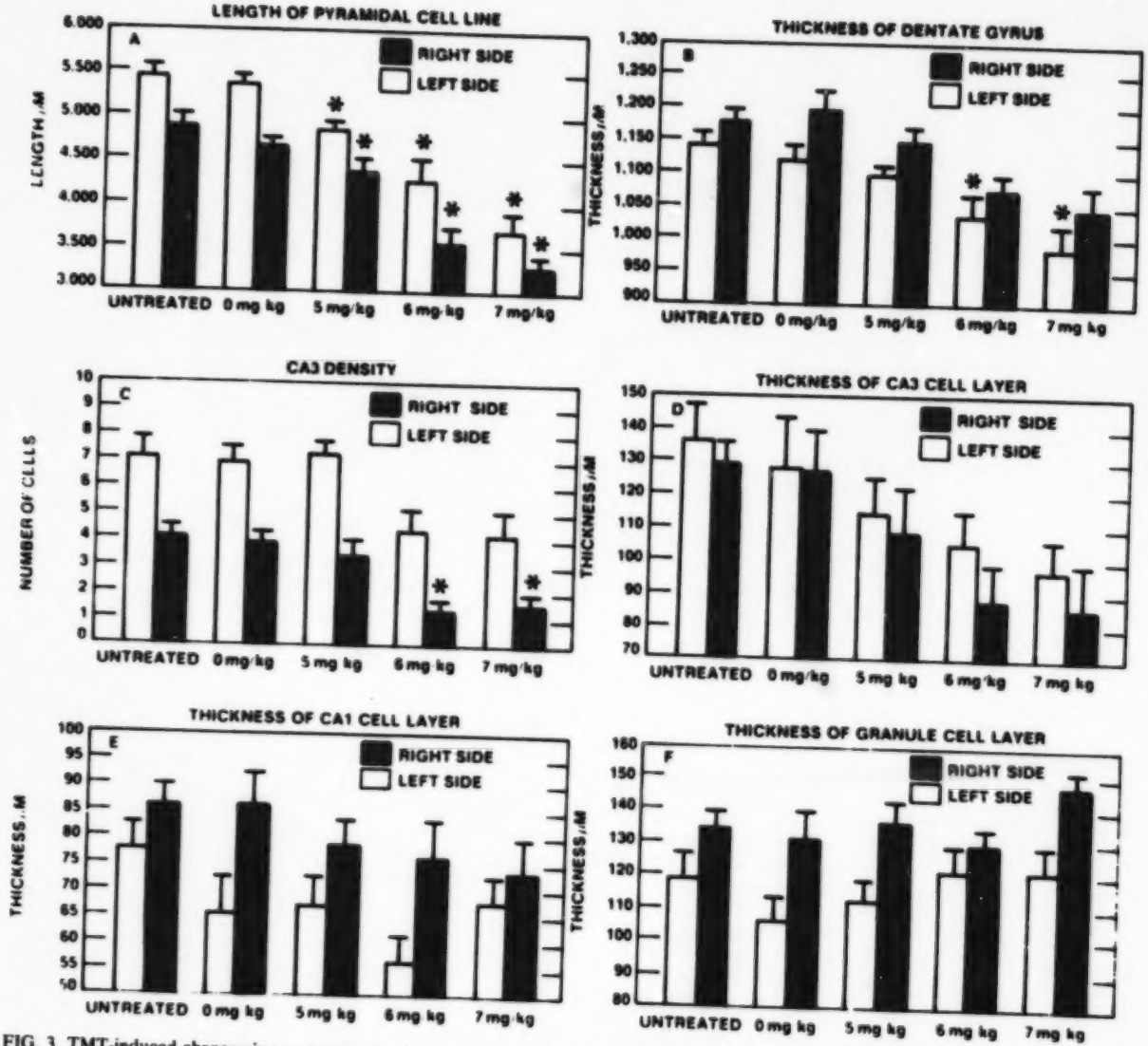

FIG. 3. TMT-induced changes in measurements obtained from sagittal sections during the first experiment (30-day post-treatment sacrifice time). Left side measurements were about 0.5 mm more lateral than right side measurements. Measurements were averaged across section number within each side. (A) length of the line of pyramidal cells; (B) thickness of the dentate gyrus; (C) CA3 density (number of cells intersected by a perpendicular drawn through CA3 at its most sparsely populated point in the elbow of the area); (D) thickness of the CA3 layer at its thickest extent; (E) thickness of CA1 at its thickest extent; (F) thickness of the granule cell layer at its thickest extent (whether dorsal or ventral blade) in the horizontal plane. *=different from same side unstimulated control ($p<0.05$, Duncan's multiple range test).

granule cell layer, thickness of the dentate gyrus, and length of the pyramidal cell line. The main effect of dosage was not tested using this collapsed set of data, since it was redundant with the first two MANOVA's. There were no dosage × side interactions (MANOVA $F(24,283)=0.66$, $p>0.89$).

In the second experiment, it was observed that length of the pyramidal cell line was decreased (by about 20%) in less than 11 days, $F(1,11)=29.29$, $p<0.0002$. Due to the small number of subjects, the different post-treatment times were collapsed to make one control and one exposed group for purposes of analysis.

In the third experiment, the effects of TMT on the hippocampus were most extensive. Figure 4 illustrates sections obtained from 1 control and 3 treated animals 105 days following treatment. As shown in Fig. 5A, the length of the pyramidal cell line was reduced by more than 50 percent. In some animals, only short segments of CA1 and Ch2 remained. The length of the cell line in these controls is longer than the controls of the first two experiments because sections were obtained from a more lateral location. The length of the line of granule cells was slightly reduced (Fig. 5A) and the intrahilar thickness was reduced by nearly 30 percent

FIG. 4. Sections obtained from 4 animals used for Experiment 3 (105-day post-treatment sacrifice time). A: section from control rat; B–D: sections from rats treated with 6 mg/kg TMT. Magnification and section location were identical in all of these pictures. Calibration bar: 0.5 mm.

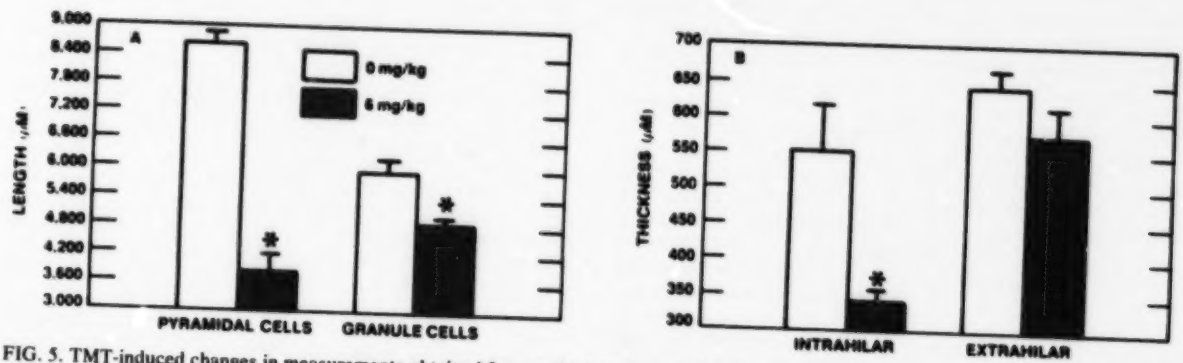

FIG. 5. TMT-induced changes in measurements obtained from sagittal sections during the third experiment (105-day post-treatment sacrifice time). (A) length of the line of pyramidal cells and granule cells; (B) thickness of the intrahilar and extrahilar region of the dentate gyrus.

(Fig. 5B). These findings were statistically significant, all F's $(1,13)>9.12$, p's<0.01. The measurements of extrahilar thickness and granule cell layer thickness obtained from the TMT-treated rats did not differ significantly from those obtained from the control rats, F's $(1,13)<4.23$, p's>0.05.

DISCUSSION

As reported by others, systemic administration of TMT produces a loss of pyramidal cells within the hippocampal formation, primarily from CA3 [2,7]. This study demonstrates quantitatively the dose-dependence and time-dependence of these changes. Following a single administration of 6 mg/kg TMT, loss of pyramidal cells appears first in CA3c, then CA3b and a. At long post-treatment intervals (105 days), damage appears more extensive but cells may remain in CA1 and the "resistant zone" (see below). The absence of differences between the two control groups in the first experiment and the absence of a treatment × side interaction in that experiment indicates that changes in hippocampal morphology were not influenced by repeated afterdischarge production or presence of an electrode.

Several differences exist between the results reported here and those reported by Brown *et al.* [2]. Brown *et al.* [2] reported a thinning of CA1, and although there was a slight thinning of the cell layer in the 30-day post-treatment study (Experiment 1), it did not approach statistical significance. It is conceivable that the difference observed by Brown *et al.* was secondary to the distance from midline effect reported here. In other words, if medial control sections were inadvertently compared to lateral treated sections, then an apparent treatment-related thinning of the CA1 cell layer might

be reported. More probably, some combination of dosage and/or post-treatment time may produce neuron loss in CA1. CA1 measurements were not made in the 105-day post-treatment study (Experiment 3), but inspection of the slides indicates that it occurred in at least some animals.

Similarly, Brown *et al.* [2] refer to a sparing of Sommer's sector by TMT. Sommer's sector is generally described as either h1 [5] or CA1 [15], an area which Brown *et al.* [2] report as thinned by TMT. Indeed the present study did find some sparing of Sommer's sector, especially at low dosages and short survival times. Brown *et al.*'s Fig. 13 [2] indicates sparing of the resistant sector of Spielmeyer [10], which we observed to be partially spared in some but not all of the brains measured in this study. The present study indicates that, at least in terms of gross cellular loss, damage begins in CA3c and progresses through CA3a and possibly into CA2, in a dosage dependent manner. Thus at low dosages Spielmeyer's zone is entirely spared, while at high dosages it may not be.

It is important to discriminate between the gross morphological assessment made in the present study and the more detailed cellular assessments made by others. Bouldin *et al.* [1] have reported that in rats the earliest TMT-induced alterations occur in the dentate granule cells. Similarly, Chang *et al.* [4] report that in the hippocampal formation of mice, the dentate granule cells are most affected. Several factors might account for the apparent discrepancy between those studies and the present study. Both studies [1,4] used electron microscopy, while the present study did not. Bouldin *et al.* [1] report that acute exposures to TMT produce more necrosis in neurons of fascia dentata than cornu ammonis, and that following chronic intoxication the reverse is true. However in their study the dosing regimen and the time course of effects are confounded. Their "acute" experiments involved repeated high dosages and short postdose sacrifice times. Thus, effects are described as acute if they follow a dose of TMT by 24 hrs. The dosing regimen is not strictly acute however since the doses were repeated. The differences which are ascribed to the differences in dosing (5 mg/kg/day for 3 or 4 days vs 1 mg/kg/2 days for 15 doses) may in fact be due simply to time since a certain fixed dosage had accumulated. Thus, if some of the acutely dosed animals had been sacrificed 30 days after the last dose, the same pattern of alterations as observed in the 30-dose experiment might have been obtained. The gross pattern of degeneration reported by Bouldin *et al.* [1] following "chronic" TMT treatment is similar to the present findings.

The third experiment in the present study does suggest some loss of dentate granule cells, presumably from the anterior ends of the blades since the line of cells was shortened. Scattered loss of cells within the dorsal and ventral blades could well have been missed due to (1) the thickness of the sections examined, (2) the dense packing of the cells, and (3) the inappropriateness of the stain and microscopic technique for demonstrating cell death. Loss of pyramidal cells was so extensive that it overcame these inadequacies. The vulnerability of CA3 pyramidal cells is not surprising. Other evidence suggests that these cells are especially vulnerable to the effects of a variety of stressors, including excess stimulation [13, 17, 19], epilepsy [5], ischemia [3], carbon monoxide hypoxia [11], dipiperidinoethane [12], and aging [9]. Indeed, the correlations between the damage induced by TMT and the damage induced by kainic acid (e.g., [14]), which is presumed to occur as a result of hyperactivation of the mossy fiber system [13], may be produced by similar mechanisms. The discrepancy between the findings of Chang *et al.* [4] and the present findings do not contradict this explanation. The differences may result from a species difference since rats and mice are known to differ markedly in their response to both kainic acid [16] and TMT (K. Hulebak, personal communication).

Finally, it should be pointed out that the measurements made in the present study are quite crude. Thus, the measurement of cell loss in specific regions was thickness of the cell layer. Although this was the apparent criterion used by Brown *et al.* [2], assessing cell loss by this method implies that either (1) cell loss occurs from one side of the line of cells or the other, and is not scattered through the layer; or (2) that loss may occur throughout the layer, but the remaining cells close up the distance. There is no a priori reason to assume that either of these two possibilities is true. Indeed, the two methods herein used to measure CA3, which showed decreased density with no decreased thickness, indicate that either cell loss is evenly distributed throughout the thickness of the layer, or at least does not occur preferentially at one side of the layer or the other.

The thinning of the dentate gyrus in the present study is demonstrated to result from intrahilar thinning. It is likely that both cell loss and fiber loss contribute to the change. CA3c cells are entirely lost from the intrahilar region, and Bouldin *et al.* [1] have described loss of small myelinated fibers within the hilus as well.

In conclusion, from the measurements obtained here it is possible to single out length of the pyramidal cell line as a simple dosage-dependent correlate of hippocampal damage induced by TMT. The data indicate that within a limited range (between 2 and 3 mm from midline) the effect of TMT is not lateral distance-specific. However the distance from midline does affect this parameter, and thus between-treatment comparisons must be made at a fixed distance from the midline. The clear dosage-dependence of the measure suggests that it may be useful as a marker for comparison with behavioral and physiological studies.

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